



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12P 7/64, A01H 5/10, C12N 9/02	A1	(11) International Publication Number: WO 97/37033 (43) International Publication Date: 9 October 1997 (09.10.97)
(21) International Application Number: PCT/SE97/00247 (22) International Filing Date: 14 February 1997 (14.02.97) (30) Priority Data: 9601236-4 29 March 1996 (29.03.96) SE (71)(72) Applicants and Inventors: BAFOR, Maureen [NG/NG]; P.O. Box 4196, Benin City (NG). BANAS, Antoni [PL/PL]; ul. Wiolinowa 14, PL-08-110 Siedlce (PL). DAHLQVIST, Anders [SE/SE]; Hemmansvägen 2, S-244 66 Furulund (SE). GUMMESON, Per-Olov [SE/SE]; Bjömbärsstigen 5, S-227 38 Lund (SE). LEE, Michael [NZ/SE]; Storgatan 11, S-231 97 Klagstorp (SE). LENMAN, Marit [SE/SE]; Revingegatan 13 A, S-223 59 Lund (SE). SJÖDAHL, Staffan [SE/SE]; Valthornsvägen 22, S-756 50 Uppsala (SE). STYMNE, Sten [SE/SE]; Torrlösa 1380, S-268 90 Svalöv (SE). (74) Agents: ALDENBÄCK, Ulla et al.; Dr. Ludwig Brann Patentbyrå AB, P.O. Box 1344, S-751 43 Uppsala (SE).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims.</i>
(54) Title: NOVEL PLANT ENZYME AND USE THEREOF		
(57) Abstract <p>The present invention relates to a novel plant enzyme called delta (12) fatty acid acetylenase. This enzyme is responsible for the conversion of fatty acids to acetylenic acids and the invention relates to production of such acids. The invention also relates to use of cDNA encoding acetylenase, preferably <i>Crepis alpina</i> delta (12) acetylenase, for transforming organisms such as oil accumulating organisms selected from the group consisting of oil crops, oleogeneous yeasts and moulds. Furthermore, the invention relates to organisms such as oil accumulating organisms transformed with acetylenase cDNA, and to oils and other acetylenic compounds from said organisms.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

NOVEL PLANT ENZYME AND USE THEREOF**Technical field**

The present invention relates to a novel plant enzyme. More specifically, the present invention relates to a method for producing acetylenic compounds in particular acetylenic fatty acids, to a cDNA encoding a plant fatty acid acetylenase, to the use of the said cDNA for transforming oil accumulating organisms for the purpose of producing acetylenic fatty acids, and to such oil accumulating organisms per se as well as oils therefrom.

Background of the invention

There is considerable interest, world-wide, in producing chemical feedstocks such as fatty acids for industrial use from renewable plant resources rather than from non-renewable petrochemicals. This concept has broad appeal for both manufacturers and consumers on the basis of resource conservation and in addition provides significant opportunities to develop new industrial crops for agriculture.

There is an enormous diversity of unusual fatty acids in oils from wild plant species which have been well characterized (see e.g. Badami & Patil, 1981). Many of these acids are of potential industrial use. This has lead to an interest in domesticating relevant plant species to enable the agricultural production of particular fatty acids. However the development of genetic engineering combined with a greater understanding of the biosynthesis of unusual fatty acids make it now possible to transfer genes coding for key enzymes, involved in the synthesis of a particular fatty acid from a wild species, to a choosen domesticated oilseed crop. In this way specific fatty acids can be produced in high purity and quantities at moderate costs.

One class of fatty acids of particular interest are the acetylenic fatty acids; consisting of an acyl chain having two

SUBSTITUTE SHEET (RULE 26)

adjacent carbon atoms linked by an acetylenic or triple bond. Because of their high reactivities they may be ideally suited for the production of coatings, plastics and lubricants. By transferring the genes responsible for the production of a specific acetylenic acid from a wild species to commercial oilseeds, or any other oil accumulating organism that can be easily multiplied, it should be possible to develop a renewable primary source of this oil containing acetylenic fatty acids for industrial uses.

Prior art

The formation of acetylenic bonds in fatty acids in mosses occurs via the subtraction of hydrogens from a double bond (Kohn et al., 1994)

Crepis species have seed oils with high contents of acetylenic acids (Badami & Patil, 1981; Hirsinger, 1991).

Summary of the invention

The present invention provides a new method of producing acetylenic fatty acids from transgenic oil accumulating organisms.

The inventors have characterized an enzyme (acetylenase) that is responsible for the production of 9-octadecen-12-ynoic acid (crepenynic acid) from 9,12-octadecadienoic acid (linoleic acid) in membrane fractions from developing *Crepis alpina* seeds. The characterization of the acetylenase from *Crepis alpina* revealed that the acetylenase had very similar biochemical properties to the non-heme containing monooxygenases oleate delta 12 and linoleate delta 15 (omega 3) desaturases. Based on the premise that the biochemical similarities observed between the acetylenase and the enzymes producing linoleic and linolenic acid (delta 12 and delta 15 desaturases) would also be associated with similarity in the

SUBSTITUTE SHEET (RULE 26)

primary sequence of these proteins a full length cDNA (pCrepl), encoding a putative acetylenase, was isolated from *Crepis alpina*.

Initially, two types of cDNA fragments, obtained by using PCR and primers designed by aligning protein sequences of delta 12 desaturases, were characterised from *C. alpina*. DNA sequence analysis revealed that one was highly homologous to all the other plant endoplasmic reticular (ER) delta 12 desaturases and the castor bean hydroxylase. The other cDNA fragment characterised had a sequence that was homologous to the ER delta 12 desaturase sequences of plants but was divergent not only in a number of non-conserved amino residues but also in a number of amino acid residues that were highly conserved in all delta 12 ER desaturases. Using northern blot analysis the gene encoding this cDNA (pCrepl) was observed to be highly expressed only in a seed specific manner when compared to expression in leaf tissue. Taken together these findings, and a consideration of the unique biochemical nature of an cell in a oilseed, provided strong evidence that the isolated cDNA (pCrepl) from *C. alpina* encode an enzyme responsible for converting linoleic acid into crepenynic acid.

Finally, conclusive evidence that the cDNA, pCrepl, from *C. alpina* encoded a plant acetylenase enzyme was obtained by the expression of this gene in yeast. The expression of this gene together with the addition of linoleic acid when culturing these yeast resulted in the production of a delta 12 acetylenic acid, 9-octadecen-12-ynoic acid (crepenynic acid), as confirmed by mass spectrometric analysis of extracted yeast fatty acids.

Therefore, in a first aspect, the present invention relates to a method of producing acetylenic compounds, characterized in that a double bond is converted to an acetylenic bond by an acetylenase.

SUBSTITUTE SHEET (RULE 26)

In a preferred embodiment of the method, the acetylenic fatty acids are produced by conversion of unsaturated fatty acids to acetylenic fatty acids by a fatty acid acetylenase.

In a second aspect, the invention relates to cDNA coding for acetylenase of the mixed function monooxygenase type containing three conserved histidin motifs (HX(3 or 4)H, HX(2 or 3)HH, and HX (2 or 3)HH) according to Fig. 1 of the accompanying drawings.

In a further embodiment the invention relates to a cDNA encoding fatty acid acetylenase, such as *Crepis alpina* delta 12 acetylenase comprising the sequence according to Fig. 3 of the accompanying drawings or any nucleotide sequences essentially homologous therewith.

A third aspect of the invention concerns use of the above described cDNA for transforming organisms. The organisms may be acetylenic compound accumulating organisms or oil accumulating organisms, respectively.

In a fourth aspect, the invention relates to organisms transformed with a acetylenase cDNA as described above. The organisms are acetylenic compound or oil accumulating, examples of the latter being oil crops, oleogeneous yeasts and moulds.

In a fifth aspect, the invention concerns acetylenic compounds accumulated in organisms described above.

In a sixth aspect, the invention concerns oils from oil accumulating organisms described above.

In a preferred embodiment, the present invention relates to transforming oil accumulating organisms with the said isolated cDNA from *Crepis alpina* seed cDNA library for the purpose of producing acetylenic fatty acids and in particular 9-octadecen-12-ynoic acid (crepenynic acid).

SUBSTITUTE SHEET (RULE 26)

Detailed description of the invention

C. alpina seed oil is rich in crepenynic acid [9-octadecen-12-ynoic acid (Hirsinger, 1989)]. The inventors have studied the biosynthesis of crepenynic acid in *C. alpina* seeds. The feeding of exogenous 1-¹⁴C-labelled free fatty acids to intact developing cotyledons of *C. alpina* seeds demonstrated that linoleate is a precursor to crepenynic acid. This is contradictory to previous published results for the biosynthesis of crepenynic acid in *Crepis rubra* (Haigh & James, 1967).

Although the reaction of acetylenic acid formation in mosses has been shown to be a desaturation process (Kohn et al. 1994), such desaturation processes can be carried out by a variety of different unrelated types of plant enzymes, such as phytoene desaturases (Wieland et al. 1994) or non-heme containing proteins, the latter a class of enzymes of which some show very little amino acid sequence homologies except for three conserved histidin motifs (Shanklin et al. 1994). It has been suggested that the biosynthesis of acetylenic fatty acids occur by a sequence of intermediates catalyzed by separate enzymatic reactions. For example, acetylenic bonds were thought to be formed as a side pathway of saturated fatty acid synthesis (Diedrich & Henschel, 1991); or via an epoxygenation of a double bond with subsequent conversion to a diol which in its turn is dehydrated in two steps in order to form an acetylenic bond (Van de Loo et al, 1993). Given these conflicting alternatives the nature of an acetylenase enzyme and its mechanism of action was not known at all nor obvious at the time of the present priority patent application SE 9601236-4.

The enzyme, according to this invention, responsible for the synthesis of crepenynic acid (called the delta 12 acetylenase), was shown by the inventors to remain only active in membrane (microsomal) fractions prepared from developing seeds of *Crepis alpina*, provided that the homogenization buffer contain NADH or NADPH, catalase and free coenzyme A. The char-

SUBSTITUTE SHEET (RULE 26)

acterisation of the microsomal acetylenase and its comparison with the delta 12 desaturase (responsible for the desaturation of oleate to linoleate) revealed that these enzymes had very similar properties. Both enzymes required O₂ and NADH or NADPH; where both coreductants worked equally well with both enzymes. Cyanide (CN⁻) and antibodies against cauliflower cytochrome b₅ inhibited both these enzymes whereas carbonmonoxide had no significant effect on either enzyme activity. These data suggested that both enzymes were biochemically similar. The oleate delta 12 hydroxylase from castor bean was also shown to have similar biochemical properties to the delta 12 desaturase despite catalyzing a different reaction (Bafor et al., 1991, Smith et al, 1992). The castor bean delta 12 hydroxylase gene was later shown to have significant sequence homology to the ER delta 12 desaturase genes (FAD 2 genes) (Van de Loo et al., 1995). Because the delta 12 acetylenase, like the delta-12 desaturase (FAD2), catalyzes a dehydrogenation between carbons 12 and 13 of an acyl chain, and like the delta 15 desaturase (FAD3) utilized linoleic acid as substrate the inventors considered the possibility that the acetylenase gene should have some sequence homology to the FAD2 and/or the FAD3 genes.

The invention will now be described more closely below in relation to the accompanying drawings and an Experimental Part.

The drawings show:

Fig. 1. Restriction map of pCrepl

Fig. 2. Restriction map of pVT-Crepl

Fig. 3. Superimposed single ion chromatograms of ions 333, 365, 367 from FADEA prepared from total fatty acids extracted from yeast strain YN94-1 transformed with pVT-Crepl. The letters denotes peaks representing the following diethylamide de-

SUBSTITUTE SHEET (RULE 26)

rivatives of fatty acids: A, eicosanoic acid; B, eicosaenoic acid; C, 9-octadecen-12-ynoic acid.

Fig. 4. Superimposed single ion chromatograms of ions 333, 365, 367 from FADEA prepared from total fatty acids extracted from yeast strain YN94-1 transformed with empty vector (pVT100U; control). The letters denotes peaks representing the following diethylamide derivatives of fatty acids: A, eicosanoic acid; B, eicosaenoic acid.

Fig. 5. A total ion chromatogramme of FADEA prepared from fatty acids enriched in the putative 9-octadecen-12 ynoic acid originating from lipid extracts of YN94-1 transformed with pVT-Crepl. The letters denotes peaks representing the following diethylamide derivatives of fatty acids: A, hecadenanoic acid; B, octadecaenoic acid; C, octadeca-9,12-dienoic acid; D. 9-octadecen-12-ynoic acid.

Fig. 6. Mass spectrum of compound corresponding to peak D in Fig.5.

EXPERIMENTAL PART

Cloning of putative acetylenase gene

An alignment of amino acid sequences from different species showed that the membrane bound fatty acid desaturases could be grouped according to the homology of their putative mature protein into three distinct groups (plastid delta 12 desaturases, ER delta 12 desaturases and delta 15 desaturases; see Sequence Listing 1). The castor bean hydroxylase (Van de Loo et al 1995) shared a high homology with the ER delta 12 desaturases to the degree that it was not easily distinguishable from these sequences. Furthermore, the sequences from all three classes of enzymes showed some degree of sequence homology with each other.

SUBSTITUTE SHEET (RULE 26)

Based on this alignment oligonucleotide primers were designed and synthesised for these three groups of sequences and for a consensus of all of these sequences. The sequence of these primers are given below.

(i) consensus primers (primers designed to a consensus of all three groups of membrane-bound desaturases and the castor bean fatty acid hydroxylase):

sense is GSN CAY GAN TGY GSN CAY

antisense is RAN ADR TGR TGN RBN AYR TG.

(ii) plastid delta 12 desaturase primers:

sense is TGG MGN TTY AAR CAY GAY MG

antisense is GTN SWC ATC CAR AAR TGR TA.

(iii) ER delta 12 desaturase primers including the castor bean fatty acid hydroxylase:

sense is CAY GAR TGY GGN CAY CAY GC

antisense is CCN CKN ARC CAR TCC CAY TC.

(iv) delta 15 desaturase primers:

sense is ACN CAY CAY CARAAAY CAY GG

antisense is CAY TGY TTN CCN CKR TAC CA.

Poly A+ RNA was isolated from developing seeds (100 mg) of *C. alpina* using a QuickPrep Micro mRNA purification kit from Pharmacia Biotech. All of the poly A+ RNA from this purification was precipitated and used to synthesise first strand cDNA which was primed with both oligo dT and random hexamers and synthesised with Superscript II reverse transcriptase from Gibco BRL. The polymerase chain reaction (PCR) was then used, with the described primers and this cDNA, to amplify products with the following cycling conditions: 1 cycle of 94°C for 2 min, 30 cycles of (94°C, 30 sec; 50°C, 30 sec; 72°C, 30 sec) and finally one cycle of 72°C for 5 min.

SUBSTITUTE SHEET (RULE 26)

Products were obtained for all the primers used; particularly noticeable was that the primers against the ER delta 12 desaturases gave significantly more product than from the other primers used. The sizes of the PCR products from the delta 12 and delta 15 primers corresponded to the sizes anticipated.

The PCR products obtained by amplification with the ER delta 12 primers and delta 15 primers were made blunt ended with T4 and klenow polymerases and cloned into the EcoRV site of the plasmid vector Bluescript. DNA sequencing of a number of the clones revealed that at least three distinct sequences had been amplified when using these two sets of primers: (i) a highly conserved delta 15 desaturase sequence (ii), a highly conserved ER delta 12 sequence and (iii) a sequence (D12V) having homology to the ER delta 12 sequences but showing distinct differences even in some amino acid residues that were highly conserved amongst all the other desaturase sequences.

The analysis of fatty acids from *C. alpina* had indicated that the crepenynic acid was probably present only in seeds. Northern blot analysis at high stringency indicated that the mRNA from the D12V sequence described above was expressed highly in seeds but not in leaves which is consistent with the observation that crepenynic acid was only observed in seeds.

A cDNA library was made from developing seeds from *C. alpina* using a Uni-ZAP XR cloning kit for cDNA from Stratagene and screened with the random labelled D12V sequence. From this screening it was estimated that cDNAs encoding the D12V sequences were highly abundant; further emphasizing the high level of expression of this gene. After the isolation of single hybridising Lambda plaques, pBluescript phagemid was excised using the ExAssist/SOLR system from Stratagene. Phagemids obtained by this were subsequently used to produce double stranded DNA plasmid. From these colonies a full length clone (pCrepl, see Fig. 1) was isolated by using DNA sequencing and restriction mapping of isolated plasmid. The insert

SUBSTITUTE SHEET (RULE 26)

from pCrepl, a 1.5 kb insert contained in the vector pBluescript SK, was sequenced and from this an open reading frame deduced coding for a 375 aa long protein (Sequence Listing 2). The analysis of this protein sequence revealed approximately 60% identity and 80% similarity with other plant delta 12 desaturase proteins and had noticeable differences in homology, where, certain residues that were conserved amongst all other desaturases were not in this sequence (see Sequence Listing 1). Three histidin motifs were present which have been shown to be conserved in a number of non-heme containing monooxygenases catalyzing hydroxylation and desaturation reactions (Shanklin et al. 1994).

Expression of the pCrep cDNA and detection of crepenynic acid in transgenic yeast

The pCrepl open reading frame was released from pCrepl on a *SmaI/XhoI* restriction fragment and the 1.5 kb *Crepl* open reading frame recovered by gelpurification (Langridge et al., 1980). pVT100-U DNA (Vernet et al., 1987) was digested using *PvuII* and *XhoI*. 50 ng *PvuII/XhoI*-linearized pVT100 was ligated with 100 ng 1.5 kb *SmaI/XhoI* fragment corresponding to the *Crepl* open reading frame using T4 DNA ligase (NBL Genen Science Ltd., UK). Part of the ligation mixture was used to transform competent *E.coli* DHa cells. One clone (pVT-Crepl), which contained the expected 1.5 kb insert, was chosen and the construct checked by digestion with *EcoRI*, or *HindIII* + *XbaI*. Both digests gave the expected products (approx. 5.3, 2.3 and 0.8 kb for the *EcoRI* digest, and release of the 1.5 kb open reading frame with the *HindIII* + *XbaI* digest). pVT-Crepl DNA (see Fig. 2), or empty vector pVT100U, was used to transform *Saccharomyces cerevisiae* strains YN94-1 and C13-ABYS86, using the PLATE method of Elble (1992). Overnight yeast transformants were spread on SCD minus uracil agar and single colonies were streaked onto fresh selective (minus uracil) plates.

SUBSTITUTE SHEET (RULE 26)

The YN94-1 and C13-ABYS86 strains of yeast transformed with pVT-Crep1 DNA and with empty vector (pVT100U; control) were cultivated in shaking cultures at 28°C for five hours in selective media (without uracil; 400 ml) whereafter 40 ml of cultivation media containing linoleic acid dispersed in Tween 40® was added to the culture to give a final concentration of 0.03% linoleic acid and 1% Tween 40® (w/w). After cultivation for an additional 78h at 28°C the cells were pelleted by centrifugation and washed by dispersion in 20 ml of 0.1M Tris-HCl buffer pH. 7.8 containing 1% Tween 40® and repelleted by centrifugation. The cells were further washed by resuspension in 20 ml of 0.1M Tris-HCl buffer pH. 7.8 and pelleted again. The cells were thereafter extracted in a mixture of chloroform/methanol/ 0.15M acetic acid (1:2:0.8 by vol.) in a Braun MSK glass bead cell homogenizer (B. Braun Biotech International, Melsungen, Germany) at 4000 r.p.m. for 20 s. The yeast lipids were extracted from the mixture into a chloroform phase by adding chloroform and 0.15M acetic acid to yield final proportions of 1:1:0.9 (by vol.) of chloroform, methanol and 0.15 M acetic acid. After centrifugation of the mixture the lipid containing chloroform phase was removed and evaporated to dryness under a stream of nitrogen.

The lipophilic residue were methylated with methanolic HCl (4% w/w) at 85°C for 45 min whereafter the fatty acid methyl esters were extracted into n-hexane. Gas liquid (GC) chromatogrammes of the methyl esters separated on a glass column (2.5m x 3 mm i.d.) containing 3% SP-2300 on Supelcoport 100/120 mesh (Supelco, Bellefonte, P. USA) revealed a peak with the same retention time as authentic 9-octadecen-12 ynoic acid methyl ester constituting up to 0.3% of total peak areas in samples prepared from yeast transformed with pVT-Crep1 but not in samples prepared from yeast transformed with empty vector (pVT100U; control).

Since acetylenic fatty acid methyl esters can be partially separated from other fatty acid methylesters on silica gel

SUBSTITUTE SHEET (RULE 26)

thin layer chromatography, the methylesters prepared from YN94-1 transformed with pVT-Crepl were separated on silica gel 60 thin layer chromatography plates (Merck, Darmstadt, Germany) by developing the plate in hexane/diethyl ether/acetic acid (85:15:1 by vol.). An area located just below the main methyl ester area was removed from the plate and the lipids were eluted with methanol/chloroform (2:1) and analyzed by gas liquid chromatography. The fraction were shown to consist of fatty acid methylesters where the peak with the same retention time as 9-octadecen-12 ynoic acid metyl ester made up 12.5% of the total peak area.

The methyl ester fraction enriched in the putative 9-octadecen-12 ynoic acid methyl ester as well as total fatty acid methyl esters prepared from YN94-1 transformed with pVT-Crepl and YN94-1 transformed with empty vector (pVT100U; control) were hydrolyzed in 2.5M KOH in aqueous methanol (15% methanol, by vol.) at 90°C for 1 h. The free fatty acids were extracted into hexane after acidification with HCl and the hexane phase was evaporated to dryness under a stream of nitrogen.

Fatty acid diethylamides (FADEA) were prepared from the free fatty acids according to Nilsson and Liljenberg (1991). The FADEA were either injected directly on a gas liquid chromatography coupled to mass spectrometer (GC-MS) or subjected to further purification by silica gel thin layer chromatography by developing the plate in heptane/diethyleter/acteic acid (50:50:1, by vol.).

The FADEA were analyzed on a Hewlett-Packard 5890 II gas chromatograph equipped with a DB225 (0.25 mm i.d. x 30 m, J&W, Folsom, USA) in series with a Rtx 2330 (Restek Corp., PA, USA) fused silica capillary column, coupled with a Hewlett-Packard 5989A mass spectrometer working in electron impact mode at 70 eV. Injection technique was cold splitless at 100°C and then the temperature was raised as quickly as possible to 240°C.

SUBSTITUTE SHEET (RULE 26)

Oven temperature was 100°C for 7 min, then 20°C per min to 190°C and then 1°C per min to a final temp. of 225°C where it was kept for another 20 min. The double bond positions were determined according to Nilsson and Liljenberg (1991).

Single ion chromatogrammes of masses corresponding to the molecular ion of FADEA prepared from total fatty acids from YN94-1 transformed with pVT-Crepl and from YN94-1 transformed with empty vector (pVT100U; control) are shown in Fig.5 and Fig.6, respectively. Chromatogram of FADEA from YN94-1 transformed with pVT-Crepl showed a peak of mass 333 (corresponding to the molecular weight of 9-octadecen-12 ynoic acid diethylamide) which was absent in the chromatogram of FADEA from YN94-1 transformed with empty vector (pVT100U; control). The peak had a retention time of 57.3 min and was located between peaks corresponding to eicosanoic and eicosenoic FADEA derivatives.

A total ion chromatogramme of FADEA prepared from fatty acids enriched in the putative 9-octadecen-12 ynoic acid by thin layer chromatography (as described above) originating from lipid extracts of YN94-1 transformed with pVT-Crepl is shown in Fig. 5. Mass spectrum (Fig.6) of the putative 9-octadecen-12 ynoic acid diethylamide derivative (peak D in Fig.5) showed a gap in mass of 26 amu instead of regular 28 between carbon 7 and 9 indicating a double bond at position 9. Further more there was a gap of 24 amu instead of regular 28 between carbon atom 10 and 12 indicating acetylenic bond at position 12. The peak D produced a mass spectrum identical to that of authentic 9-octadecen-12 ynoic acid diethylamide prepared from oils from *Crepis alpina* seeds. Thus the peak D in the chromatogram in Fig 5 was unambiguously identified as 9-octadecen-12 ynoic acid diethylamide derivative. Since the compound was absent in yeast strains not transformed with the Crepl cDNA it is clear that the Crepl cDNA codes for a delta-12 fatty acid acetylenase.

SUBSTITUTE SHEET (RULE 26)

REFERENCES

Badami, R.C., and Patil, K.B. (1981). Structure and occurrence of unusual fatty acids in minor seed oils. *Progress in Lipid Research*, 19, 119-53.

Banas, A., Bafor, M., Wiberg, E., Lenman, M., Ståhl, U. and Stymne, S. (1997) Biosynthesis of an acetylenic fatty acid in microsomal preparations from developing seeds of *Crepis alpina*. In: Williams, J.P., Mobasher, K.U., Lem, N.W. (eds) *Physiology, biochemistry and molecular biology of plant lipids*. Kluwer Academic Publisher, Dordrecht. *In-press*

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513-1523

Diedrich, M. & Henschel, K.P. (1991) The natural occurrence of unusual fatty acids: Part 3. Acetylenic fatty acids. *Nahrung* 35, 193-202

Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. *Biotechniques* 13, 18-20

Haigh, W.G. & James, A.T. *Biochim. Biophys. Acta* 137, 391-392

Hirsinger, F. (1989). New annual oil crops. In *Oil crops of the world*, (eds. G. Röbbelen, R.K. Downey and A. Ashri), pp.518-532. Mc-Graw-Hill Inc.

Kohn, G., Hartmann, E., Stymne, S. & Beutelmann, P. (1994) Biosynthesis of acetylenic acids in the moss *Ceratodon purpureus*. *J. Plant Physiol.* 144, 265-271

SUBSTITUTE SHEET (RULE 26)

Langridge, J., Langridge, P and Bergquist P.L. (1980) Extraction of nucleic acids from agarose gels. Anal. Biochem. 103, 264-271

Nilsson, R. and Liljenberg, C. (1991) The determination of double bond positions in polyunsaturated fatty acids - Gaschromatography/ mass spectrometry of the diethylamide derivative. Phytochemical analysis 2, 253-259

Shanklin, J., Whittle, E. & Fox, B.G. (1994) Eight histidine residues are catalytically essential in membrane -associated iron enzyme, stearyl-CoA desaturase and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33, 12787-12794.

Stymne, S. & Lenman, M. (1996) Novel plant enzyme and use thereof. Swedish patent application no. 9601236-4, 96-03-29.

Vernet, T., Dignard, D. and Thomas, D.Y. (1987) A family of yeast expression vectors containing the phafe f1 intergenic region. Gene 52, 225-233

Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, G., Lechner, M., Bravo, J.M., Poralla, K. & Goetz, F. (1994) Genetic and biochemical analysis of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of *Staphylococcus aureus*. J. Bacteriol. 176, 7719-7726.

van de Loo, F.J., Broun, P., Turner, S. & Somerville, C. (1995) An oleate D12-hydroxylase from *Ricinus communis* L. is a fatty acid acyl desaturase homolog. Proc. Natl. Acad. Sci. USA 95, 6743-6747.

van de Loo, F.J., Fox, G. & Somerville, C. (1993) Unusual fatty acids. In: T.S. Moore Jr (ed.) Lipid Metabolism in Plants. CRC Press, Boca Raton, page 105.

SUBSTITUTE SHEET (RULE 26)

INFORMATION ABOUT SEQUENCE LISTING NO 1

Alignment of amino acid sequences from delta 12 ER and plastid desaturases, delta 15 desaturases and from the castor bean hydroxylase. Also included in this alignment is the protein sequence derived from pCrep1 (crepis). Underlined are three histidin motifs that are conserved in non-heme containing monooxygenases.

Sequences given in this alignment together with their accession numbers are : bnom6des.seq, delta 12 desaturase from *Brassica napus* (L29214);
gmom6des.seq, delta 12 desaturase from *Glycine max* (L29215);
atom3des.seq, delta 15 desaturase from *Arabidopsis thaliana* (L22961);
bnom3des.seq, delta 15 from *Brassica napus* (L22963);
rcom3des.seq, delta 15 desaturase from *Ricinus communis* (L25897);
siom3des.seq, delta 15 desaturase from oriental sesame (U25817);
ldd15des.seq, delta 15 desaturase from *Limnanthes douglasii* (U17063);
gsom3des, delta 15 desaturase from *Glycine max* (L22965);
atom3bdes.seq, delta 15 desaturase from *Arabidopsis thaliana* (D17579);
bnom31des.seq, delta 15 from *Brassica napus* (L22962);
gsom3bdes.seq, delta 15 desaturase from *Glycine max* (L22964);
atd12des.seq, delta 12 desaturase from *Arabidopsis thaliana* (L26296);
gmom6bdes.seq delta 12 desaturase from *Glycine max* (L43921);
scom12des.seq, delta 12 desaturase from *S. commersonii* (X92847);
gmom6ades.seq, delta 12 desaturase from *Glycine max* (L43920);
rchyd.seq, oleate 12-hydroxylase from *Ricinus communis* (U22378);
crepis, *Crepis alpina* acetylenase from this document.

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING 1

	1				50
bnom6des.seq	MASRIA	DSLFAFTGPQ QCLPRAPKLA
gmom6des.seq	MACTLA	DSLLEFKGSY Q.KPVLRRDI
atom3des.seq	.MANLVLSEC	GIRPLPRIYT	TPRSNFLSNN	N...KFRPSL	SSSSYKTSSS
bnom3des.seq
rcom3des.seq	MAAGWVLSEC	GLRPLPRIYS	RPRIGFTSKT	TNLLKLRELP	DSKSYNLCSS
siom3des.seq	.MASWVLSEC	GLRPLPRVYP	KPRTGHPLLN	SNPTKLRFER	TDLGNGSS..
ldd15des.seq	.MASWVLSQY	ALNPLPHIFR	TPRTSITSHKL	TVSHTNNRAT
gsom3des.seq	.MATWYHQKC	GLKPLAPVIP	RPRTGAALSS	TSRVEF....	..LDTNKKVVA
atom3bdes.seq
bnom3l1des.seq
gsom3bdes.seq
atd12des.seq
gmom6bdes.seq
scom12des.seq
gmom6ades.seq
rchyd.seq
crepis
	51				100
bnom6des.seq	SARLSPGVYA	VRPIDLLLKG	TRRTFLVPAK	KRIGCIKAVF	VPVAPPSADN
gmom6des.seq	AARYSPGIFS	LNSNGLIQKR	FRQRNFVTR	NKVTVIHAVA	IPVQPAPVES
atom3des.seq	PLSFGLNSRD	GFTRNWALNV	STPLTTPIFE	ESP.....LEEDNK
bnom3des.seq
rcom3des.seq	FKVSSWSNSK	QSNWALNVAV	PVNVSTVSGE	DDREREEFNG	IVN.VDEGKG
siom3des.seq	...FCLSSGI	LREKNWALRV	SAPLRVLQVE	EEEEENKEGER	VIN.GGEE..
ldd15des.seq	PDLTKLSLIK	FRERKLGLRV	SAPFQIASTT	PE.....EEDEV
gsom3des.seq	GPKFQPLRCN	LRERNWGLKV	SAPLRVASIE	EEQKSVDLTN	GTNGVEHEKL
atom3bdes.seq	MVV	AMDQRTNVNG DPGAGDRKKE
bnom3l1des.seq	MVV	AMDQRSNANG D.....
gsom3bdes.seq	MV	KDTKPLAYAA NNGYQQKGSS
atd12des.seq	MGAG	GRMPVP.... TSSKKSETDT
gmom6bdes.seq	MGAG	GRTDVP.... PANRKSEVDP
scom12des.seq	MGAG	GRMSAP.... NGETEVKRNP
gmom6ades.seq	MGLAKETTMG	GRGRVA.... KVEVQGK.KP
rchyd.seq	MGGG	GRMSTVITSN NSEKKGSSSH
crepis	MGGG	GR..... ..GRTSQKPL
	101				150
bnom6des.seq	AEDREQLAES	YGFKQIGQDL	PDNVTCLKDIM	DTLPKEVFEI	DDVKAWKSVL
gmom6des.seq	AEYRKQLAED	YGFRQVGEPL	SDDVTCLKDVI	NPLPKEVFEI	DDVKAWKSVL
atom3des.seq	QRFDPGAPPP	FNLADIRAAI	PKHCWVKNPW	KSLSYVVRDV	AIVFA.....
bnom3des.seqMSYVREL AIVFA.....
rcom3des.seq	EFFDAGAPPP	FTLADIRAAI	PKHCWVKNPW	RSMSYVLRDV	VVVF.....
siom3des.seq	..FDPGAPPP	FKLSDIREAI	PKHCWVKDPW	RSMGYVVRDV	AVVFG.....
ldd15des.seq	AEFDPSGPPP	FKLADIRAAI	PKHCWVKQW	RSMSYVVRDV	VIVLG.....
gsom3des.seq	PEFDPGAPPP	FNLADIRAAI	PKHCWVKDPW	RSMSYVVRDV	IAVFG.....
atom3bdes.seq	ERFDPSAQPP	FKIGDIRAAI	PKHCWVKSPL	RSMSYVVRDI	IAVAA.....
bnom3l1des.seq	ERFDPSAQPP	FKIGDIRAAI	PKHCWVKSPL	RSMSYVARDI	FAVVA.....
gsom3bdes.seq	FDFDPSAPPP	FKIAEIRASI	PKHCWVKNPW	RSLSYVLRDV	LVIAA.....
atd12des.seq	TKRVPCEKPP	FSVGDLLKAI	PPHCFKRSIP	RSFSYLISDI	IIASC.....
gmom6bdes.seq	LKRVPFEKPQ	FSLSQIKKAI	PPHCFQRSVL	RSFSYVVDL	TIAFC.....
scom12des.seq	LQKVPTSKPP	FTVGDIKKAI	PPHCFQRSIL	RSFSYVVDL	ILVSI.....
gmom6ades.seq	LSRVPNTKPP	FTVGQLKKAI	PPHCFQRSLL	TSFSYVVDL	SFAF.....
rchyd.seq	LKRAPHTKPP	FTLGDLKRAI	PPHCFERSFV	RSFSYVAYDV	CLSFL.....
crepis	MERVSVD.PP	FTVSDLKQAI	PPHCFKRSVI	RSSYYIVHDA	IIAYI.....

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING 1 (cont.)

	151				200
bnom6des.seq	ISVTSYALGL	FMIAPAPWYL	LPLAWAWTGT	AVTGFFVIGH	DCAHKSFSKN
gmom6des.seq	ISVTSYALGL	FMISKAPWYL	LPLAWVWTGT	AITGFFVIGH	DCAHRSFSSN
atom3des.seq	LAAGAAAYL..NNWIV	WPLYWLAQGT	MFWALFVLGH	DCGHGSFSND
bnom3des.seq	LAAGAAAYL..NNWLIV	WPLYWIAQGT	MFWALFVLGH	DCGHGSFSND
rcom3des.seq	LAAVAAYF..NNWVA	WPLYWFCQGT	MFWALFVLGH	DCGHGSFSNN
siom3des.seq	LAAVAAYF..NNWVV	WPLYWFAQST	MFWALFVLGH	DCGHGSFSND
ldd15des.seq	LAAAAYL..NSWAV	WPLYWVAQGT	MFWALFVLGH	DCGHGSFSNN
gsom3des.seq	LAAAAYL..NNWLIV	WPLYWAAQGT	MFWALFVLGH	DCGHGSFSNN
atom3bdes.seq	LAIAAVYV..DSWFL	WPLYWAAQGT	LFWAIFVLGH	DCGHGSFSDI
bnom3l1des.seq	LAVAAVYF..DSWFF	WPLYWAAQGT	LFWAIFVLGH	DCGHGSFSDI
gsom3bdes.seq	LVAAAIHF..DNWLL	WLIYCPIQGT	MFWALFVLGH	DCGHGSFSDS
atd12des.seq	FYYVATNYFS	LLPQPLSYLA	WPLYWACQGC	VLTGIWVIAH	ECGHHAFFSDY
gmom6bdes.seq	LYYVATHYFH	LLPGPLSFRG	MAIYWAVQGC	ILTGWVIAH	ECGHHAFFSDY
scom12des.seq	MYVANTYFH	LLPSPYCYIA	WPIYWICQGC	VCTGIWVNAH	ECGHHAFFSDY
gmom6ades.seq	IFYIATTYFH	LLPQPFLIA	WPIYWVLQGC	LLTGWVIAH	ECGHHAFFSKY
rchyd.seq	FYSIATNFFP	YISSPLSYVA	WLWVWLFQGC	ILTGLWVIGH	ECGHHAFFSEY
crepis	FYFLADKYIP	ILPAPLAYLA	WPLYWFCQAS	ILTGLWVIGH	ECGHHAFFSDY
	201				250
bnom6des.seq	KLVEDIVGTL	AFLPLVYPYE	PWRFKHDRHH	AKTNMLVHDT	AWQPVPPPEEF
gmom6des.seq	KLVEDIVGTL	AFMPLIYPYE	PWRFKHDRHH	AKTNMLREDT	AWHPVWKDEF
atom3des.seq	PKLNSVVGHL	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWHPMSEKIY
bnom3des.seq	PRLNSVVGHL	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWHPMSEKIY
rcom3des.seq	PKLNSVVGHL	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWHPLSEKIY
siom3des.seq	PKLNSVVGHL	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWHPLSEKIY
ldd15des.seq	HKLNSVVGHL	LHSSILVPYH	GWRIRHRTTH	QNHGHVENDE	SWHPMSEKLF
gsom3des.seq	SKLNSVVGHL	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWHPLPEKLF
atom3bdes.seq	PLLNSVVGHI	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWVPLPERVY
bnom3l1des.seq	PLLNTAVGHI	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWVPLPEKLY
gsom3bdes.seq	PLLNSLVGHI	LHSSILVPYH	GWRISHRTHH	QNHGHVENDE	SWVPLPEKLY
atd12des.seq	QWLDDTVGLI	FHSFLLVPYF	SWKYSHRRHH	SNTGSLERDE	VFVPPKQKSAI
gmom6bdes.seq	QLLDDIVGLI	LHSALLVPYF	SWKYSHRRHH	SNTGSLERDE	VFVPPKQKSCI
scom12des.seq	QWVDDTVGLI	LHSALLVPYF	SWKYSHRRHH	SNTGSLERDE	VFVPPKPKSQL
gmom6ades.seq	QWVDDVVGIL	LHSTLLVPYF	SWKISHRRHH	SNTGSLDRDE	VFVPPKPKSKV
rchyd.seq	QLADDIVGLI	VHSALLVPYF	SWKYSHRRHH	SNIGSLERDE	VFVPPKPKSKI
crepis	QWVDDTVGFI	LHSFLMTPYF	SWKYSHRNHH	ANTNSLDNDE	VYIPKSKAKV
	251				300
bnom6des.seq	DS.....	.SPVLRKAI	FGYGPIRPWL	SI.....AH	WVNWHFNLRK
gmom6des.seq	ES.....	.TPLLRKAI	YGYGPFRCWM	SI.....AH	WLMWHFDLKK
atom3des.seq	NTLDK....	PTRFFRFTLP	LVMLAYPFYL	WARSPGKK..	..GSHYHPDS
bnom3des.seq	KSLDK....	PTRFFRFTLP	LVMLAYPFYL	WARSPGKK..	..GSHYHPDS
rcom3des.seq	KSLDN....	VTKTLRFSLP	FPMLAYPFYL	WSRSPGKK..	..GSHFHPDS
siom3des.seq	KNLDT....	ATKKLRFTLP	FPLLAYPIYL	WSRSPGKQ..	..GSHFHPDS
ldd15des.seq	RSLDK....	IALTFRFKAP	FPMLAYPFYL	WERSPGKT..	..GSHYHPDS
gsom3des.seq	RSLDT....	VTRMLRFETAP	FPLLAFFVYL	FSRSPGKT..	..GSHFDPSS
atom3bdes.seq	KKLPH....	STRMLRYTVP	LPMLAYPLYL	CYRSPGKE..	..GSHFNPYS
bnom3l1des.seq	KNLSH....	STRMLRYTVP	LPMLAYPLYL	WYRSPGKE..	..GSHYNPYS
gsom3bdes.seq	KNLDS....	MTRLIRFTVP	FPLFVYPIYL	FSRSPGKE..	..GSHFNPYS
atd12des.seq	KWYGKYLNNP	LGRIMMLTVQ	F.VLGWPLYL	AFNVSGRPYD	GFACHFFPNA
gmom6bdes.seq	KWYSKYLNNP	PGRVLTAVT	L.TLGWPLYL	ALNVSGRPYD	RFACHYDPYG
scom12des.seq	GWYSKYLNNP	PGRVLSLTIT	L.TLGWPLYL	AFNVSGRPYD	RFACHYDPYG
gmom6ades.seq	AWFSKYLNNP	LGRAVSLVT	L.TIGWPMYL	AFNVSGRPYD	SFASHYHPYA
rchyd.seq	SWYSKYNNP	PGRVLTAAAT	L.LLGWPLYL	AFNVSGRPYD	RFACHYDPYG
crepis	ALYYKVLNHP	PGRLIMFIT	F.TLGFPLYL	FTNISGKKYE	RFANHFDPMS

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING 1 (cont.)

	301				350
bnom6des.seq	..FRPSEVNR	VKISLACVFA	FMAVGWPLII	YKVGVLGWVK	FWLMPWLGYH
gmom6des.seq	..FRPSEVPR	VKISLACVFA	FIAIGWPLII	YKTGIMGWIK	FWLMPWLGYH
atom3des.seq	DLFLPKERKD	VLSTACWTA	.MAALLVCLN	FTIGPIQMLK	LYGIPYWINV
bnom3des.seq	DLFLPKERND	VLSTACWTA	.MAVLLVCLN	FVMGPMQMLK	LYVIPYWINV
rcom3des.seq	GLFVPKERKD	IITSTACWTA	.MAALLVYLN	FSMGPVQMLK	LYGIPYWIFV
siom3des.seq	DLFVPNEKKD	VITSTVCWTA	.MLALLVGLS	FVIGPVQLLK	LYGIPYLGNV
ldd15des.seq	DLFVPSEKKD	VITSTICWTT	.MVGLLIIGLS	FVMGPIQILK	LYVVPYWIFV
gsom3des.seq	DLFVPNERKD	VITSTACWAA	.MLGLLVGLG	FVMGPIQLLK	LYGVPIYVIFV
atom3bdes.seq	SLFAPSERKL	IATSTTCWSI	.MFVSLIALS	FVFGPLAVLK	VYGVPIIIFV
bnom3lides.seq	SLFAPSERKL	IATSTTCWSI	.MLATLVYLS	FLVGPTVLK	VYGVPIIIFV
gsom3bdes.seq	NLFPPSERKG	IAISTLCWAT	.MFSLLIYLS	FITSPLLVK	LYGIPYWIFV
atd12des.seq	PIYNDRERLQ	IYLS DAGILA	.VCFGLYRYA	AAQGMASMIC	LYGVPLLIIVN
gmom6bdes.seq	PIYSDRERLQ	IYISDAGVLA	.VVYGLFRLA	MAKGLAWVVC	VYGVPLLVVN
scom12des.seq	PIYNNRERLQ	IFISDAGVLG	.VCYLLYRIA	LVKGLAWLVC	VYGVPLLVVN
gmom6ades.seq	PIYSNRERLL	IYVSDVALFS	.VTYSLYRVA	TLKGLVWLLC	VYGVPLLIIVN
rchyd.seq	PIFSEERERLQ	IYIADLGIFA	.TTFVLYQAT	MAKGLAWVMR	IYGVPLLIIVN
crepis	PIFKERERFQ	VLLSDLGLLA	.VLYGVKLAV	AAKGAAWVTC	IYGIPVLGVF
	351				400
bnom6des.seq	FWMSTFTMVH	HTAPH..IPF	KPADEWNAAQ	AQLNGTVHCD	YPSWIEILCH
gmom6des.seq	FWMSTFTMVH	HTAPY..IPF	KYSEEWNRAQ	AQLNGTVHCD	YPKWIEILCH
atom3des.seq	MWLDFTVYLH	HHGHEDKLPW	YRGKEWSYLR	GGL.TTLDRD	YGLINNIHHD
bnom3des.seq	MWLDFTVYLH	HHGHEDKLPW	YRGKEWSYLR	GGL.TTLDRD	YGLINNIHHD
rcom3des.seq	MWLDFTVYLH	HHGHEDKLPW	YRGKAWSYLR	GGL.TTLDRD	YGWINNIHHD
siom3des.seq	MWLDLVTYLH	HHGHEDKLPW	YRGKEWSYLR	GGL.TTLDRD	YGWINNIHHD
ldd15des.seq	MWLDFTVYLD	HHGHEDKLPW	YRGKEWSYLR	GGL.TTLDRD	YGLINNIHHD
gsom3des.seq	MWLDLVTYLH	HHGHEDKLPW	YRGKEWSYLR	GGL.TTLDRD	YGWINNIHHD
atom3bdes.seq	MWLDAVTYLH	HHGHDEKLPW	YRGKEWSYLR	GGL.TTIDRD	YGI FNNIHHD
bnom3lides.seq	MWLDAVTYLH	HHGHDDKLPW	YRGKEWSYLR	GGL.TTIDRD	YGI FNNIHHD
gsom3bdes.seq	MWLDFTVYLH	HHGHHQKLPW	YRGKEWSYLR	GGL.TTVDRD	YGWINYNIHHD
atd12des.seq	AFLVLITYLQ	H..THPSLPH	YDSSEWDWLR	GAL.ATVDRD	YGI LNKVFHN
gmom6bdes.seq	GFLVLITFLQ	H..THPALPH	YTSSEWDWLR	GAL.ATVDRD	YGI LNKVFHN
scom12des.seq	GFLVLITYLQ	H..THPSLPH	YDSTEWDWLR	GAL.ATCDRD	YGV LNKVFHN
gmom6ades.seq	GFLVTITYLQ	H..THFALPH	YDSSEWDWLK	GAL.ATMDRD	YGI LNKVFHH
rchyd.seq	CFLVMITYLQ	H..THPAIPR	YGSSEWDWLR	GAM.VTVDRD	YGV LNKVFHN
crepis	IFFDIITYLH	H..THLSLPH	YDSSEWNWLR	GAL.STIDRD	FGFLNSVLHD
	401				450
bnom6des.seq	DINVHIPHHI	SPRIPSYNLR	AAHQSIQENW	GKYTNLATWN	WRLMKTIMTV
gmom6des.seq	DINVHIPHHI	SPRIPSYNLR	AAHKSLOENW	GQYLNEASWN	WRLMKTIMTV
atom3des.seq	I.GTHVIHHL	FPQIPHYHLV	EATEAAKPVL	GKYYREPDKS	.GPLPLHLL
bnom3des.seq	I.GTHVIHHL	FPQIPHYHLV	EATEAAKPVL	GKYYREPDKS	.GPLPLHLLG
rcom3des.seq	I.GTHVIHHL	FPQIPHYHLV	EATEAAKPM	GKYYREPKKS	.GPLPLHLLG
siom3des.seq	I.GTHVIHHL	FPQIPHYHLI	EATEAAKPVL	GKYYREPKKS	.APLPFHLLG
ldd15des.seq	I.GTHVIHHL	FPQIPHYHLV	EATQAAPKIF	GKYYKEPAKS	.KPLPFHLID
gsom3des.seq	I.GTHVIHHL	FPQIPHYHLV	EATEAAKPVF	GKYYREPKKS	AAPLPFHLLG
atom3bdes.seq	I.GTHVIHHL	FPQIPHYHLV	DATKAACHVL	GRYYREP KTS	.GAIPHLVE
bnom3lides.seq	I.GTHVIHHL	FPQIPHYHLV	DATKSAKHVL	GRYYREP KTS	.GAIPHLVE
gsom3bdes.seq	I.GTHVIHHL	FPQIPHYHLV	EATQAAPKVL	GDYYREPERS	.APLPFHLLK
atd12des.seq	ITDTHVAHHL	FSTMPHYNAM	EATKAIPIL	GDYYQFDGTPWYV
gmom6bdes.seq	ITDTHVAHHL	FSTMPHYHAM	EATKAIPIL	GEYYRFDETPFVK
scom12des.seq	ITDTHVHHL	FSTMPHYNAM	EATKAVKPLL	GDYYQFDGTPIYK
gmom6ades.seq	ITDTHVAHHL	FSTMPHYHAM	EATNAIKPIL	GEYYQFDDTPFYK
rchyd.seq	IADTHVAHHL	FATVPHYHAM	EATKAIPIM	GEYYRYDGTFYK
crepis	VTHTHVMHHL	FSYIPHYHAK	EARDAINTVL	GDFYKIDRTILK

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING 1 (cont.)

	451				493
bnom6des.seq	CHVYDKEENY	IPFDRLAPEE	SQPITFLKKA	MPDYAA....	...
gmom6des.seq	CQVYDKEKSL	CCLRRTCP..
atom3des.seq	ILAKSIKEDH	YV.....SDE	GEVVYYKADP	NLYGEVKVRA	D..
bnom3des.seq	ILAKSIKEDH	FV.....SDE	GDVVYYEADP	NLYGEIKVTA	E..
rcom3des.seq	SLVRSMKEDH	YV.....SDT	GDVVYYQKDP	KLSGIGGEKT	E..
siom3des.seq	DLTRSLKRDH	YV.....SDV	GDVVYYQTD	QLTGAEKS..	...
ldd15des.seq	VLLKSLKRDH	FV.....PDT	GDIVYYQSDP	QISGSLKPE.	...
gsom3des.seq	EIIRSFKTDH	FV.....SDT	GDVVYYQTD	KINGSSKLE.	...
atom3bdes.seq	SLVASIKKDH	YV.....SDT	GDIVFYETDP	DLYVYASDKS	KIN
bnom31des.seq	SLVASIKKDH	YV.....SDT	GDIVFYETDP	DLYVYASDKS	KIN
gsom3bdes.seq	YLIQSMRQDH	FV.....SDT	GDVVYYQTD	LLLHSQRD..	...
atd12des.seq	AMYREAKECI	YVEPDREGDK	KGVIWYNNKL
gmom6bdes.seq	AMWREARECI	YVEPDQSTES	KGVIWYNNKL
scom12des.seq	EMWREAKECL	YVEKDESSQG	KGVIWYNNKL
gmom6ades.seq	ALWREARECL	YVEPDGTSE	KGVIWYNNKY
rchyd.seq	ALWREARECL	FVEPDGAPT	QGVFWYNNKY
crepis	AMWREAKECI	FIEPEKGRES	KGVIWY.NKF

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING 2

Nucleotide sequence and derived amino acid sequence of the open reading frame from plasmid pCrepl.

```
ATGGGTGGCGGTGGCCGTGGTTCGGACTTCGCAAAAACCCCTCATGGAACGTGTCTCAGTT
M G G G G R G R T S Q K P L M E R V S V

GATCCACCCTTCACCGTGAGTGATCTCAAGCAAGCAATCCCTCCCCATTGCTTCAAGCGA
D P F F T V S D L K Q A I P P H C F K R

TCTGTAATCCGTTCTCTTACTACATAGTCCACGATGCTATTATCGCCTACATCTTCTAC
S V I R S S Y Y I V H D A I I A Y I F Y

TTCCTTGCCGACAAATACATTCCGATTCTCCCTGCCCCTCTAGCCTACCTCGCTTGGCCC
F L A D K Y I P I L P A P L A Y L A W P

CTTTACTGGTTCTGTCAAGCTAGCATCCTCACCGGCTTATGGGTCATCGGTACGAATGC
L Y W F C Q A S I L T G L W V I G H E C

GGTCACCATGCCTTCAGCGACTACCAAGTGGGTTGACGACACTGTGGGCTTCATCCTCCAC
G H H A F S D Y Q W V D D T V G F I L H

TCGTTTCTCATGACCCCGTATTTCTCCTGGAAATACAGCCACCGGAACCACCATGCCAAC
S F L M T P Y F S W K Y S H R N H H A N

ACAAATTGCTTGACAACGATGAAGTTTACATCCCCAAAAGCAAGGCCAAAGTCGCGCTT
T N S L D N D E V Y I P K S K A K V A L

TACTATAAAGTTCTCAACCACCCACCTGGCCGACTGTTGATTATGTTTCATCACCTTCACC
Y Y K V L N H P P G R L L I M F I T F T

CTAGGCTTCCCTCTATACCTCTTTACCAATATTTCCGGCAAGAAGTATGAAAGGTTTGCC
L G F P L Y L F T N I S G K K Y E R F A

AACCATTTGACCCCATGAGTCCGATTTTCAAAGAGCGTGAGCGGTTTCAGGTCTTGCTA
N H F D P M S P I F K E R E R F Q V L L

TCGGATCTTGCCCTTCTTGCTGTGCTTTACGGAGTTAAACTTGCGGTAGCAGCGAAAGGC
S D L G L L A V L Y G V K L A V A A K G

GCCGCTGGGTGACGTGCATTTACGGAATTCAGTTTTAGGCGTGTTTATCTTTTTTCGAT
A A W V T C I Y G I P V L G V F I F F D

ATCATCACCTACTTGCACCACACCCATCTGTCGTTGCCTCATTATGATTCATCTGAATGG
I I T Y L H H T H L S L P H Y D S S E W

AACTGGCTCAGAGGGGCTTTGTCAACAATCGATAGGGACTTTGGGTTCTGAATAGTGTG
N W L R G A L S T I D R D F G F L N S V

CTCCATGATGTTACACACACTCACGTTATGCATCATCTGTTTTATACATTCCACACTAT
L H D V T H T H V M H H L F S Y I P H Y

CATGCGAAGGAGGCAAGGGATGCAATCAACACAGTCTTGGGCGACTTTTATAAGATCGAT
H A K E A R D A I N T V L G D F Y K I D

AGGACTCCAATTCTGAAAGCAATGTGGAGAGAGGCCAAGGAATGCATCTTCATCGAGCCT
R T P I L K A M W R E A K E C I F I E P

GAAAAAGGTAGGGAGTCCAAGGGTGTATATTGGTACAATAAATTCTGA
E K G R E S K G V Y W Y N K F *
```

SUBSTITUTE SHEET (RULE 26)

CLAIMS

1. A method of producing acetylenic compounds, characterized in that a double bond is converted to an acetylenic bond by an acetylenase.
2. A method according to claim 1, wherein acetylenic fatty acids are produced by conversion of unsaturated fatty acids to acetylenic fatty acids by a fatty acid acetylenase.
3. A method according to claim 2, wherein C18 fatty acids with doublebonds at position delta 12 are converted to 12-ynoic acids.
4. A method according to claim 3, wherein linoleic acid is converted to crepenynic acid (9-octadecen-12-ynoic acid) by *Crepis alpina* delta 12 acetylenase.
5. cDNA coding for acetylenase of the mixed function monooxygenase type containing three conserved histidin motifs (HX(3 or 4)H, HX(2 or 3)HH, and HX (2 or 3)HH) according to Sequence Listing 1.
6. cDNA according to claim 5 encoding fatty acid acetylenase.
7. cDNA according to claim 6 encoding *Crepis alpina* delta 12 acetylenase comprising the sequence according to Sequence Listing 2 or any nucleotide sequences essentially homologous therewith.
8. Use of cDNA according to any of the claims 5, 6 or 7 for transforming organisms.
9. Use according to claim 8, wherein the organisms will be capable of accumulating acetylenic compound.

RECTIFIED SHEET (RULE 91)

10. Use according to claim 8, wherein the organisms are oil accumulating organisms.
11. Use according to claims 10, wherein the oil accumulating organisms are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.
12. Organisms transformed with a acetylenase cDNA according to any of the claims 5, 6 or 7.
13. Organisms according to claim 12, which are organisms accumulating acetylenic compounds.
14. Organisms according to claim 12, which are organisms accumulating oil.
15. Organisms according to claim 14, which are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.
16. Acetylenic compounds accumulated in organisms according to claim 13.
17. Oils from oil accumulating organisms according to claims 14 or 15.

SUBSTITUTE SHEET (RULE 26)

AMENDED CLAIMS

[received by the International Bureau on 30 July 1997 (30.07.97);
original claims 1-17 replaced by amended claims 1-18 (2 pages)]

1. A method of producing acetylenic compounds, characterized in that C18 fatty acids with doublebonds at position delta 12 are converted to 12-ynoic acids by an acetylenase.
2. A method according to claim 1, wherein linoleic acid is converted to crepenynic acid (9-octadecen-12-ynoic acid) by *Crepis alpina* delta 12 acetylenase.
3. A DNA sequence coding for acetylenase of the mixed function monooxygenase type containing three conserved histidin motifs (HX(3 or 4)H, HX(2 or 3)HH, and HX (2 or 3)HH) according to Sequence Listing 1.
4. A DNA sequence according to claim 3 encoding fatty acid acetylenase.
5. A DNA sequence according to claim 4 encoding a delta 12 fatty acid acetylenase.
6. A DNA sequence according to claim 5 encoding *Crepis alpina* delta 12 acetylenase comprising the sequence according to Sequence Listing 2 or any nucleotide sequences encoding an acetylenase essentially homologous therewith.
7. Use of a DNA sequence according to any of the claims 3, 4, 5 or 6 for transforming organisms.
8. Use according to claim 7, wherein the organisms will be capable of accumulating acetylenic compound.
9. Use according to claim 7, wherein the organisms are oil accumulating organisms.

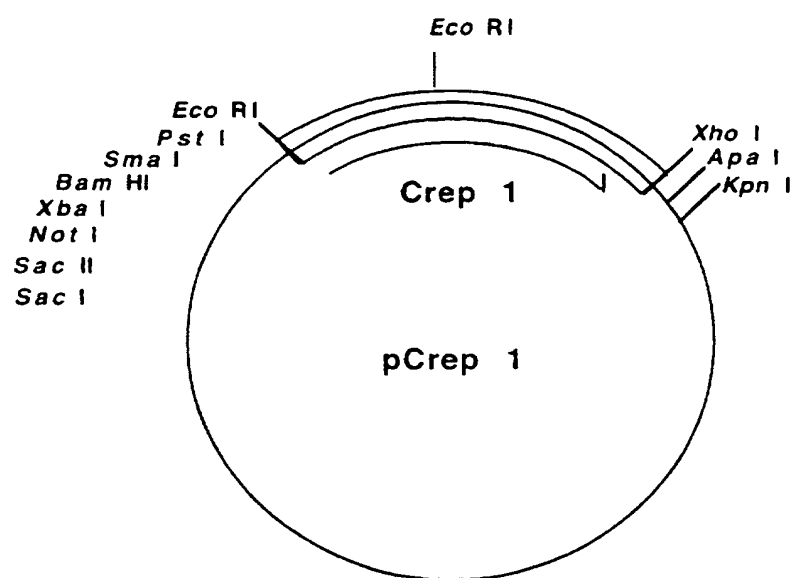
AMENDED SHEET (ARTICLE 19)

10. Use according to claim 9, wherein the oil accumulating organisms are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.
11. Organisms transformed with an acetylenase DNA according to any of the claims 3, 4, 5 or 6.
12. Organisms according to claim 11, which are organisms accumulating acetylenic compounds.
13. Organisms according to claim 11, which are organisms accumulating oil.
14. Organisms according to claim 13, which are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.
15. A method of obtaining acetylenic compounds, comprising accumulation of acetylenic compounds in organisms according to claim 12.
16. A method of obtaining oils, comprising accumulation of oils in organisms according to claims 13 or 14.
17. Acetylenic compounds obtainable by the method according to claim 15.
18. Oils obtainable by the method according to claim 16.

AMENDED SHEET (ARTICLE 19)

1/6

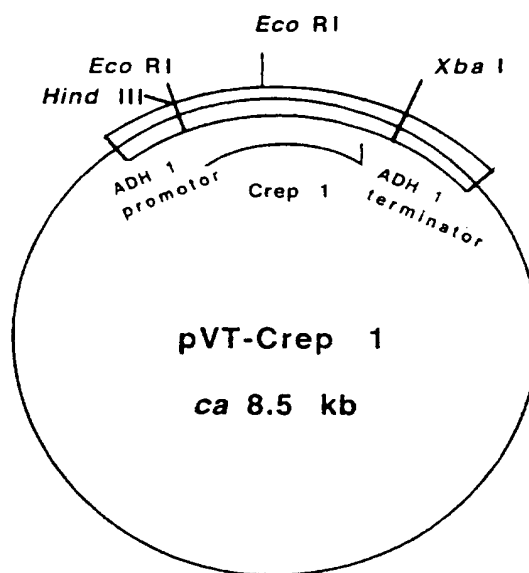
FIG. 1



SUBSTITUTE SHEET (RULE 26)

2/6

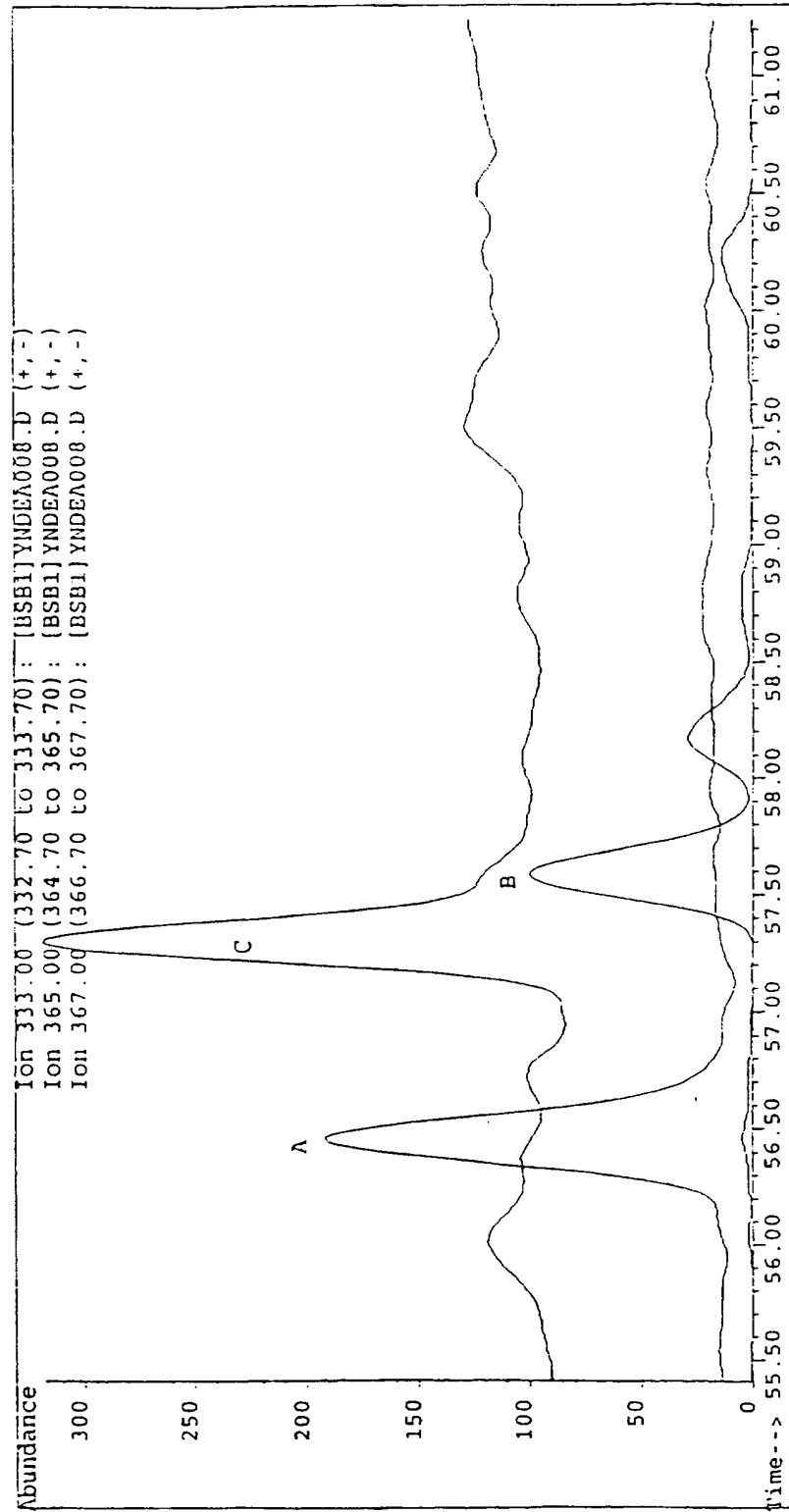
FIG. 2



SUBSTITUTE SHEET (RULE 26)

3/6

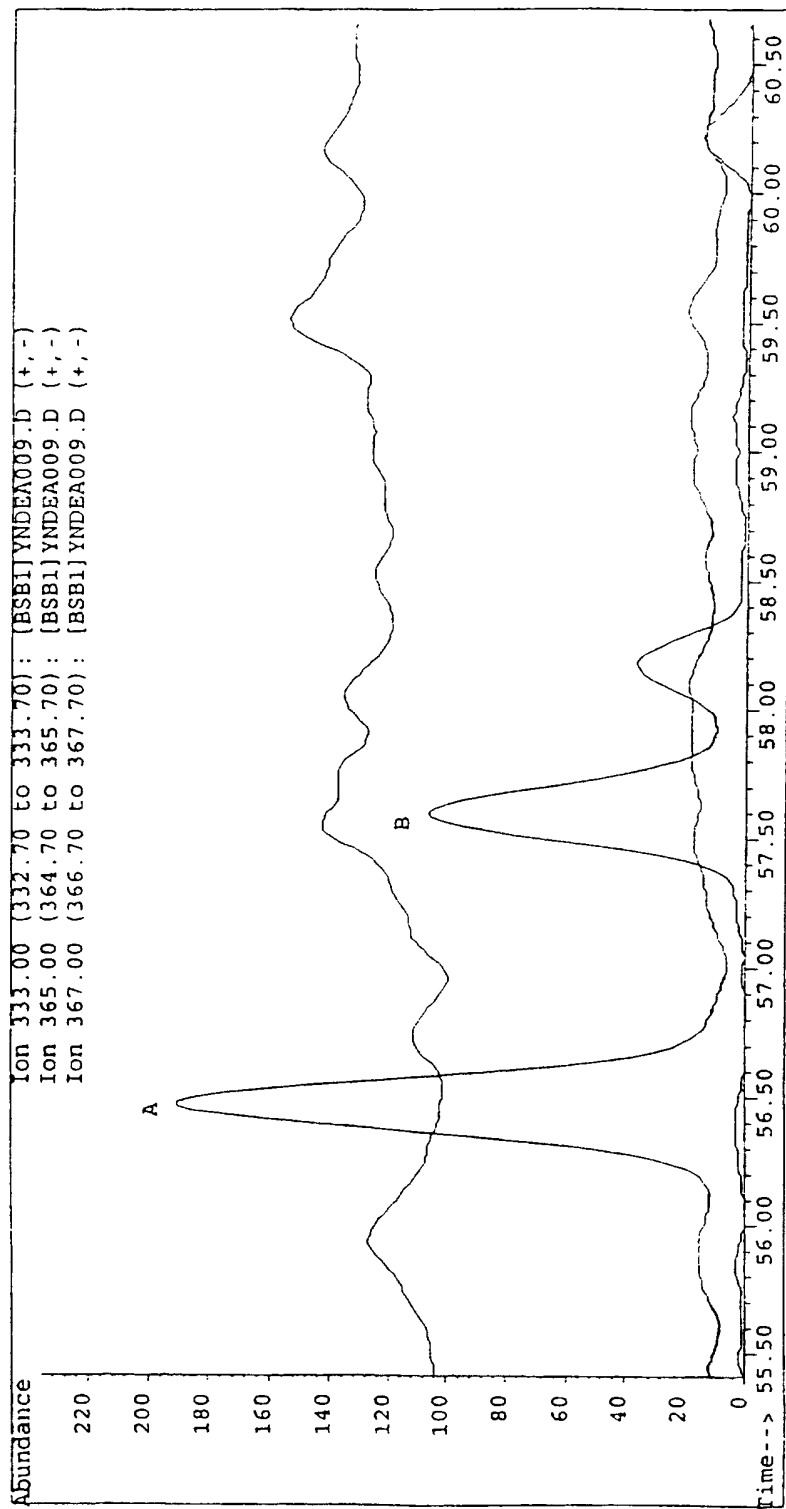
FIG. 3



SUBSTITUTE SHEET (RULE 26)

4/6

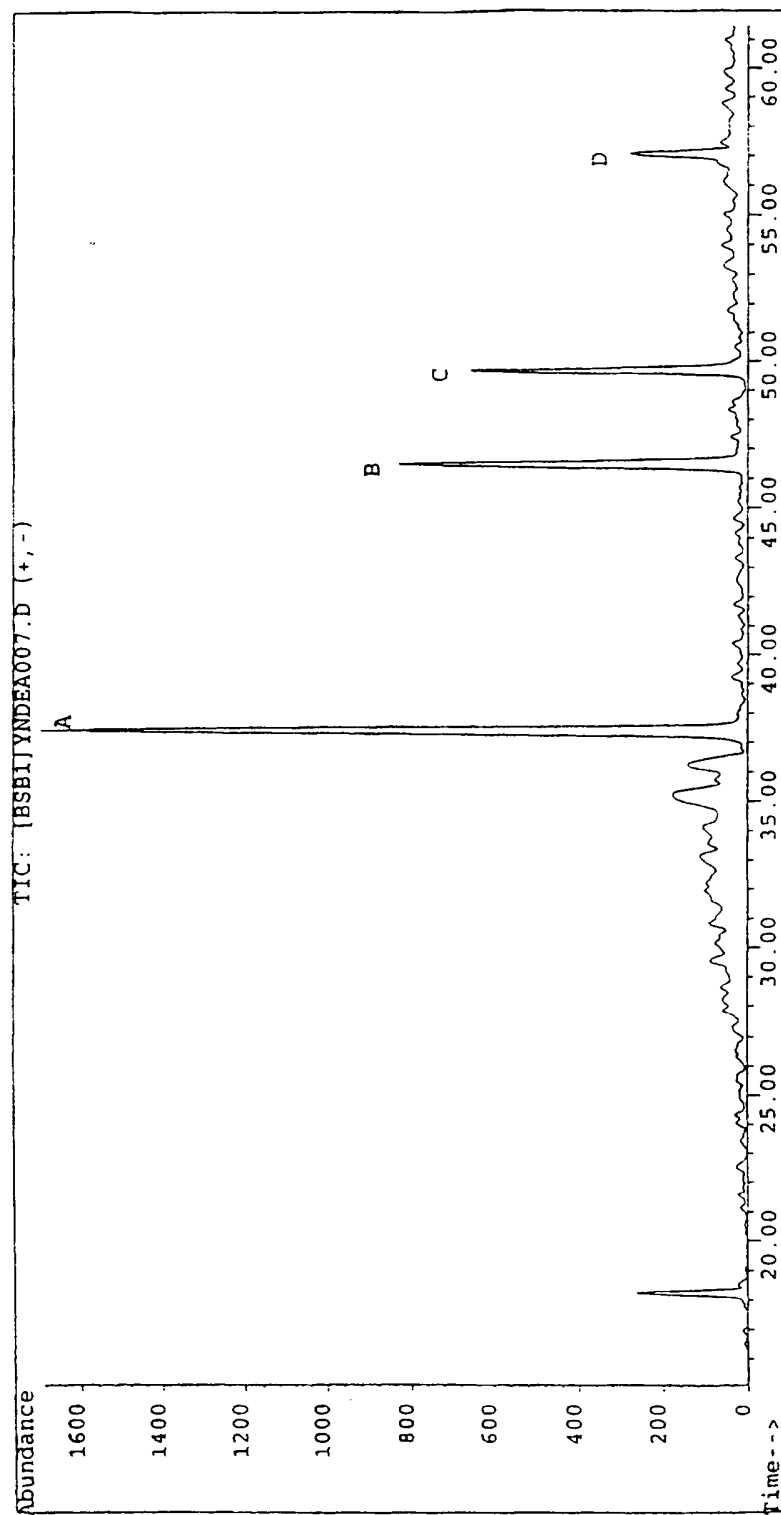
FIG. 4



SUBSTITUTE SHEET (RULE 26)

5/6

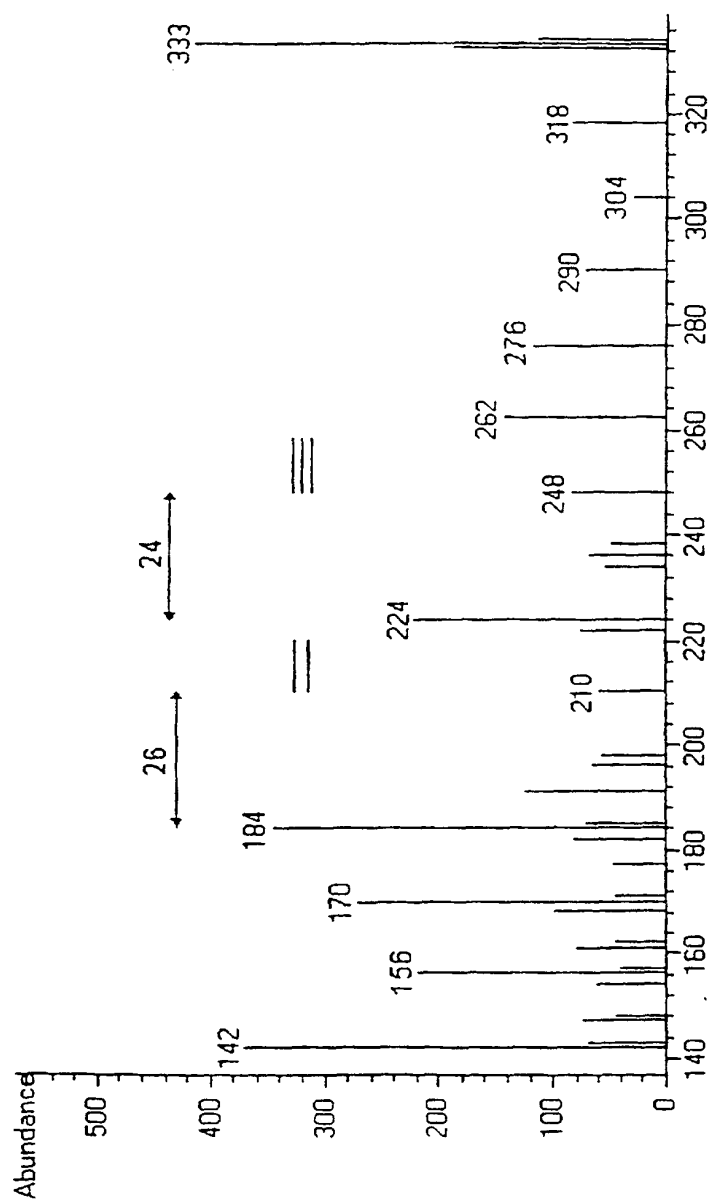
FIG. 5



SUBSTITUTE SHEET (RULE 26)

6/6

FIG. 6



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/00247

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 7/64, A01H 5/10, C12N 9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Plant Physiol., Volume 144, 1994, Gerhard Kohn et al, "Biosynthesis of Acetylenic Fatty Acids in the Moss Ceratodon purpureus (Hedw.) Brid" page 265 - page 271 --	1-2
X	Lipids, Volume 3, No 4, 1968, W.G. Haigh et al, "Acetylenic Acid Biosynthesis in Crepis rubra" page 307 --	16-17
X	Biochim. Biophys. Acta, Volume 137, 1967, Bu'Lock, "The biosynthesis of an acetylenic acid, crepenynic acid" page 391 - page 392 -- -----	16-17

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

20 May 1997

30-05-1997

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Gómez Lagerlöf
Telephone No. +46 8 782 25 00